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Microplate Technique to Determine Hemolytic Activity for Routine Typing of *Listeria* Strains

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Because the hemolysis produced by *Listeria monocytogenes* and *Listeria seeligeri* on blood agar is frequently difficult to interpret, we developed a microplate technique for the routine determination of hemolytic activity with erythrocyte suspensions. This microtechnique is a simple and reliable test for distinguishing clearly between hemolytic and nonhemolytic strains and could be used instead of the CAMP (Christie–Atkins–Munch-Petersen) test with *Staphylococcus aureus* in the routine typing of *Listeria* strains. Furthermore, our results suggest that the quantitation of the hemolytic activity of the *Listeria* strains, along with the D-xylose, L-rhamnose, and α-methyl-D-mannoside acidification tests, allows the differentiation of *L. monocytogenes*, *L. seeligeri*, and *Listeria ivanovii*. We also observed that the treatment of erythrocytes with crude exosubstances of *Rhodococcus equi*, *Pseudomonas fluorescens*, *Acinetobacter calcoaceticus*, and *S. aureus* enhanced the hemolytic activity of all *Listeria* strains with this characteristic.

The species *Listeria monocytogenes* as defined in the 8th edition of *Bergey's Manual of Determinative Bacteriology* (28) has been divided in recent years into several species. First, *Listeria innocua* was established (24) to designate the nonhemolytic and experimentally apathogenic strains, possessing antigenic O factors XI and XV (26), frequently isolated from healthy carriers and from environmental sources, to avoid confusion with the classical *L. monocytogenes* (both hemolytic and pathogenic) (23, 33). On the other hand, "*Listeria bulgarica*" was proposed to include the strains belonging to serotype 5 (13), specially pathogenic for pregnant ewes (12) and easily recognizable by their ability to produce strong bizonal hemolysis on sheep blood agar (27, 30).

Therefore, it was crucial to determine the hemolytic character of *Listeria* isolates to establish correctly their role in the epidemiology of listeriosis, in which nonhemolytic strains (apathogenic) are irrelevant (23). However, the interpretation of this characteristic on blood agar poses several problems. In the case of *L. monocytogenes*, its hemolytic effect is of variable intensity (14, 30, 31), and some strains produce such weak hemolysis that this ability may pass unnoticed. On the other hand, *L. innocua* strains may induce false-positive reactions when grown on blood agar with glucose (18, 32). The CAMP (Christie–Atkins–Munch-Petersen) reaction (6) was proposed to resolve these disadvantages because hemolysis by *L. monocytogenes* is enhanced when it is grown close to *Staphylococcus aureus* (5, 11). Other investigators, based on the observations of Fraser (10), have proposed the use of hemolytic synergism with *Rhodococcus equi* (formerly "*Corynebacterium equi*") as a reliable method to distinguish questionable or weak hemolysis by *L. monocytogenes* (17, 29, 30). In addition to hemolytic ability, other phenotypic markers, such as xylose and rhamnose acidification, were investigated to differentiate pathogenic and nonpathogenic strains without animal experimentation (3, 11).

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Recently, DNA hybridization studies were undertaken to resolve the genomic and phenotypic heterogeneity of the strains classified as *L. monocytogenes*, as defined by Seeliger and Welshimer (28) (*L. monocytogenes* sensu lato), and to determine the validity of the proposed *L. innocua* and "*L. bulgarica*" species (20). As a result of these studies, the strains included in *L. monocytogenes* sensu lato are now distributed in five species, being differentiated on the basis of hemolysis in the CAMP test with *S. aureus* and *R. equi* and acid production from D-xylose (and L-rhamnose and α-methyl-D-mannoside) (22): *L. monocytogenes* (sensu stricto), hemolytic (CAMP positive with *S. aureus*), pathogenic, xylose-negative strains; *Listeria seeligeri* (21), hemolytic (CAMP positive with *S. aureus*), experimentally nonpathogenic for mice (19), xylose-positive strains; *Listeria ivanovii* (definitive denomination of "*L. bulgarica*" strains) (25), hemolytic (CAMP positive with *R. equi*), pathogenic, xylose-positive strains; *L. innocua*, nonhemolytic and nonpathogenic, xylose-negative strains; *Listeria welshimeri* (21), nonhemolytic and nonpathogenic, xylose-positive strains.

Because hemolytic activity is a fundamental criterion for the differentiation of *Listeria* species and the interpretation of this characteristic is sometimes difficult, we propose a new method to improve the distinction between hemolytic and nonhemolytic *Listeria* strains which could be used instead of the CAMP test with *S. aureus*. We also report the effect of treatment of erythrocytes with crude exosubstances (CE) of different bacteria on hemolysis by *Listeria* strains.

MATERIALS AND METHODS

Bacterial strains and media. We studied 52 *Listeria* strains, of which 44 were *L. monocytogenes* sensu lato; of these, 13 were from the Special *Listeria* Culture Collection (SLCC), Institut für Hygiene und Mikrobiologie, Würzburg Universität, Würzburg, Federal Republic of Germany (Se-1/2a, -1/2b, -1/2c, -3a, -3b, -3c, -4a, -4b, -4ab, -4c, -4d, -5, and -7), 3 were from I. Ivanov, Central Veterinary Institute, Sofia, Bulgaria (Iv-1, -4b, and -6), 9 were provided by E. Gómez-Mampaso, Servicio de Microbiología, Hospital

TABLE 1. Titers of the different bacterial CE and treatment doses for HRBC

Source of CE	Hemolytic titer after incubation at[a]:		CE treatment dose (ml/10 ml of HRBC)
	37°C	4°C	
S. aureus	2 CHU 32 MHU	8 CHU 512 MHU	0.15 (0.5 MHU)
R. equi	64 MEHU	64 MEHU	0.80 (5 MEHU)
P. fluorescens		16 MEHU	2.50 (4 MEHU)
A. calcoaceticus		16 MEHU	2.50 (4 MEHU)

[a] Mixtures were incubated for 1 h at 37°C followed by 2 h at 4°C.

Ramón y Cajal, Madrid, Spain (GM-1 to -9), and were isolated from human coprocultures and vaginal samples, and 19 were isolated in our laboratory from milk (7) (L-16, -23, -26, -27, -123, -157, -448a, -973, -1284, -1362, -1453, -1501, and -1550, all lysotyped by J. Rocourt, Unité d'Ecologie Bactérienne, Institut Pasteur, Paris, France) and ruminant coprocultures (9) (DR-1 to -6). The remaining organisms, used as controls, were type strains (Collection de l'Institut Pasteur, Paris, France) of each of the five species defined according to the genomic groups of Rocourt et al. (22) (*L. monocytogenes* CIP 7831, *L. ivanovii* CIP 7842, *L. seeligeri* CIP 100/100, *L. innocua* CIP 8011, and *L. welshimeri* CIP 8149). We also studied one *Listeria grayi* strain (from SLCC) and two *Listeria murrayi* strains (from SLCC and I. Ivanov). The strains were subjected to the classical criteria for identification (28). D-Xylose, L-rhamnose, and α-methyl-D-mannoside acidification tests were performed by the technique described previously (22).

The strains used for treatment of erythrocytes were *R. equi* CIP 5869, *Pseudomonas fluorescens* (isolated in our laboratory), *Acinetobacter calcoaceticus* ATCC 23055, and beta-hemolytic *S. aureus* (also isolated in our laboratory).

All strains were stored at −30°C in an appropriate medium (10 g of tryptone, 20 g of powdered skim milk, 80 ml of glycerol, 320 ml of distilled water). They were grown at 37°C for 24 h on Lemco-yeast agar without glucose and with 5% sheep blood.

Hemolytic activity on blood agar. For measurement of hemolytic activity on blood agar, results were registered as follows: ++++, strong hemolysis; +++ and ++, moderate hemolysis; +, weak hemolysis; (+), questionable; −, negative. Sometimes it was necessary to remove the colony from the medium to determine hemolytic ability.

Quantitation of hemolytic activity by the microplate method. A suspension in 1% saline solution (ss) (10^{10} to 10^{11} bacteria per ml; in practice, two loopfuls in 0.5 ml of ss) was prepared from a blood agar *Listeria* culture; twofold dilutions were made in a microtiter plate with U-form wells in which 50 μl of ss had been placed. To each bacterial dilution, 100 μl of a 3% suspension of washed human erythrocytes (HRBC) (to which 10 ml of a 10% gelatin solution with 0.43% sodium azide had been added per liter) was added. The plates were incubated at 37°C for 6 to 8 h. The hemolytic activity titer was expressed as complete hemolysis units (CHU; the reciprocal of the highest dilution at which 100% hemolysis took place) and minimal hemolysis units (MHU; the reciprocal of the highest dilution at which hemolysis was detected).

(This microtechnique has a simplified version [8]. A loopful of listeria is suspended in 50 μl of ss placed in one well; 100 μl of HRBC is then added, and the mixture is incubated as described above. The results are recorded as follows: strong [including complete hemolysis], moderate, weak, negative. With this semiquantitative method a large number of strains can be studied in each plate because only one well per strain is required.)

Hemolytic activity on HRBC treated with *R. equi*, *P. fluorescens*, *A. calcoaceticus*, and *S. aureus* exosubstances. The same *Listeria* suspension used for the quantitation of hemolytic activity on HRBC was used to determine hemolytic activity by the microtechnique on HRBC treated with bacterial CE. CE were prepared by centrifuging shaken cultures (for 24 h at 37°C) of the aforementioned strains in brain heart infusion broth at 16,000 × *g* for 15 min. These CE were stored at −30°C before use. The activity of the CE of the different bacteria was determined as follows. The beta-hemolytic activity of *S. aureus* was measured by mixing 1 ml of HRBC with 1 ml of serial twofold dilutions of the CE. The mixture was incubated in a water bath for 1 h at 37°C followed by 2 h at 4°C. Beta-hemolytic activity was expressed as CHU and MHU. The hemolytic activity of the CE of *S. aureus* was also recorded after 1 h at 37°C (Table 1). Because the CE of *R. equi*, *P. fluorescens*, and *A. calcoaceticus* were nonhemolytic, their activity was determined by their ability to interact with the hemolytic effect of crude staphylococcal β-hemolysin. Serial twofold dilutions of the CE of the microorganisms in 1 ml of ss were prepared. To each dilution, 1 ml of HRBC was added. After the mixtures were incubated in a water bath at 37°C for 30 min, 35 μl of *S. aureus* CE (approximately 0.5 MHU at 37°C) was added. The mixtures were incubated for 1 h at 37°C and then cooled at 4°C for 2 h. The results obtained after incubation for 1 h at 37°C and after cooling were registered and expressed as minimal enhancing hemolysis units (MEHU).

For the tests with *Listeria* suspensions, 10 ml of HRBC was treated with 0.150, 0.8, 2.5, and 2.5 ml of the CE of *S. aureus*, *R. equi*, *A. calcoaceticus*, and *P. fluorescens*, respectively, which corresponded to approximately 0.5 MHU of the CE of *S. aureus* and 4 or 5 MEHU of the CE of the remaining bacteria (Table 1). Treated HRBC were immediately used after being prepared, and treated HRBC controls were run for quantitation of the synergistic effect on the hemolysis of the *Listeria* strains.

RESULTS

Hemolytic activity of *Listeria* strains and typing. Results of sugar acidification tests and quantitation of hemolytic activity with the microtechnique for strains belonging to *L. monocytogenes* sensu lato are shown in Tables 2 and 3.

The hemolytic activity of *L. monocytogenes* sensu stricto strains on sheep blood agar was variable. Sometimes it was marked (Se-3a), whereas for other strains (Se-4d, Iv-6, L-1362, L-23, L-26, and DR-5) it was weak, questionable, or even nonexistent. On the other hand, the microtechnique allowed us to unequivocally demonstrate this characteristic in all strains classified as *L. monocytogenes*. These strains usually showed similar hemolytic patterns (complete hemolysis in well 1 or 2 and partial hemolysis up to well 4 or 5; this corresponded to means of 6 CHU and 64.13 MHU). Nevertheless, some variations were observed; strains Se-1/2b and Se-3a showed more marked hemolysis (24 CHU for both and 192 and 384 MHU, respectively), whereas the hemolytic activity of strains Se-3b, Se-4b, Iv-4b, and L-973 was weaker (0 CHU and 24 MHU).

TABLE 2. Typing of hemolytic *Listeria* strains by sugar acidification tests and quantitation of hemolytic activity by the microtechnique and effect of treatment of HRBC with CE of different bacteria on hemolytic activity

Listeria species and strain	Sugar acidification[a]			Hemolytic activity on sheep blood agar[b]	Titer of hemolytic activity by microtechnique on:									
					HRBC[c]		HRBC treated with CE of:							
							R. equi[d]		*P. fluorescens*[e]		*A. calcoaceticus*[f]		*S. aureus*[g]	
	X	R	mM		CHU	MHU	CHU	MHU	CHU	MHU	CHU	MHU	CHU	MHU
L. monocytogenes														
CIP 7831	−	+	+	+	3	12	3	48	3	48	3	24	3	48
DR-1	−	+	+	+	3	24	12	96	6	384	3	96	6	96
DR-2	−	+	+	++	3	24	6	96	6	96	3	96	12	96
DR-3	−	+	+	+	6	48	6	96	6	96	3	192	12	96
DR-4	−	+	+	+	6	48	6	96	6	384	3	192	12	96
DR-5	−	+	+	−	6	48	6	96	6	384	6	96	12	96
DR-6	−	+	+	+	6	48	6	96	12	384	6	192	12	192
Iv-1	−	+	+	+	6	96	48	1,536	48	1,536	24	1,536	24	
Iv-4b	−	+	+	++	0	24	3	96	3	192	3	192		
Iv-6	−	+	+	(+)	3	24	6	384	12	384	6	192	6	96
L-1284	−	+	+		3	48	6	768	96	1,536	3	384	12	384
L-1362	−	+	−	(+)	6	48	3	192	24	384	3	96	6	96
L-1501	−	+	+		3	48	6	192	96	768	3	192	6	192
L-23	−	+	+	(+)	6	48								
L-26	−	+	+	(+)	6	24								
L-27	−	+	+	++	6	48								
L-488a	−	+	+	++	12	96	24	192	48	192	6	192	24	384
L-973	−	+	+		0	24	3	384	96	384	0	96	6	96
Se-1/2a	−	+	+	++	3	48	12	384	12	768	12	384		
Se-1/2b	−	+	+	++	24	192	48	768	48	384	48	384	96	
Se-1/2c	−	+	+	++	6	96	24	384	24	192	24	384	48	
Se-3a	−	+	+	+++	24	384	192	1,536	96	1,536	192	1,536	384	
Se-3b	−	+	+	+	0	24	6	384	3	384	3	96		
Se-3c	−	+	−	++	6	96	24	1,536	24	768	24	768	24	
Se-4a	−	+	+	+	12	96	48	384	24	384	12	192	96	
Se-4b	−	+	+	+	0	24	6	192	3	192	3	96		
Se-4c	−	+	+	++	6	48	12	384	12	1,536	6	192	12	384
Se-4d	−	+	+	(+)	3	24	12	768	48	1,536	6	192	12	
Se-7	−	+	+	+	6	48	12	384	24	192	12	192	24	384
L. ivanovii														
CIP 7842	+	−	−	++++	24	384	96	768	96	768	96	384	96	768
Se-5	+	−	−	+++	24	384	96	1,536	96	1,536	48	768	96	384
L. seeligeri														
CIP 100/100	+	−	−	(+)	0	3	0	24	0	48	0	24	0	3
L-123	+	−	−	(+)	0	3	0	48	0	96	0	48	0	6
L-1453	+	−	−	+	0	6	0	48	0	96	0	48	0	12
L-1550	+	−	−	(+)	0	6	0	24	0	48	0	24	0	12
L-16	+	−	−	−	0	6							0	12

[a] X, D-Xylose; R, L-rhamnose; mM, α-methyl-D-mannoside.
[b] The rating system is described in the text.
[c] 3% suspension. Means: *L. monocytogenes* strains, 6 CHU and 64.14 MHU; *L. seeligeri* strains, 0 CHU and 4.8 MHU.
[d] Means: *L. monocytogenes* strains, 20.77 CHU and 441.23 MHU; *L. seeligeri* strains, 0 CHU and 36 MHU.
[e] Means: *L. monocytogenes* strains, 30.23 CHU and 577.84 MHU; *L. seeligeri* strains, 0 CHU and 72 MHU.
[f] Means: *L. monocytogenes* strains, 16.04 CHU and 314.77 MHU; *L. seeligeri* strains, 0 CHU and 36 MHU.
[g] Means: *L. monocytogenes* strains, 38.60 CHU and 182.40 MHU; *L. seeligeri* strains, 0 CHU and 9 MHU.

The two *L. ivanovii* strains displayed very intense hemolytic activity, more intense than that produced by *L. monocytogenes* strains (except in the two aforementioned cases) both on blood agar and with the microtechnique; complete hemolysis was observed up to well 4, and partial hemolysis was observed up to well 8 (24 CHU and 384 MHU).

Both the control strain CIP 100/100 and the four strains isolated by us and typed as *L. seeligeri* manifested very weak hemolytic activity by the microtechnique; only a low degree of hemolysis could be seen in well 1 or 2 (mean of 4.8 MHU).

None of the *L. innocua*, *L. welshimeri*, *L. grayi*, or *L. murrayi* strains evidenced any hemolytic activity by the microtechnique. One of the *L. innocua* strains showed questionable hemolysis on blood agar.

In the sugar acidification tests, none of the *L. monocytogenes* strains split D-xylose, and all acidified L-rhamnose; all except two (93.1%) produced acid from α-methyl-D-mannoside, as did the control strain CIP 7831. All the *L. ivanovii* and *L. seeligeri* strains split D-xylose but not L-rhamnose or α-methyl-D-mannoside. Nonhemolytic *L. monocytogenes* sensu lato strains that did not acidify D-xylose were classified as *L. innocua*; all of these strains produced acid from α-methyl-D-mannoside, and all except two (81.8%) produced acid from L-rhamnose. Of the 11 nonhemolytic *L. monocytogenes* sensu lato strains, 1 acidified D-xylose (GM-4) and

TABLE 3. Typing of nonhemolytic *Listeria* strains by sugar acidification tests and determination of hemolytic activity by the microtechnique[a]

Listeria species and strain	Sugar acidification[b]		
	X	R	mM
L. innocua			
CIP 8011	−	+	+
GM-1	−	+	+
GM-2	−	+	+
GM-3	−	+	+
GM-5	−	+	+
GM-6	−	+	+
GM-7	−	−	+
GM-8	−	−	+
GM-9	−	+	+
L-157	−	+	+
Se-4ab	−	+	−
L. welshimeri			
CIP 8149	+	−	+
GM-4	+	+	+

[a] None of the *L. innocua*, *L. welshimeri*, *L. grayi*, or *L. murrayi* strains showed any hemolytic activity with the microtechnique on HRBC or HRBC treated with the CE of *R. equi*, *P. fluorescens*, *A. calcoaceticus*, or *S. aureus*. One of the *L. innocua* strains (L-157) showed questionable hemolytic activity on sheep blood agar; all other strains were negative.

[b] Abbreviations are defined in Table 2, footnote *a*.

was classified, therefore, as *L. welshimeri*. This strain differed from the control strain CIP 8149 in that it did produce acid from L-rhamnose.

Hemolytic activity on HRBC treated with CE of different bacteria. The hemolytic activity of *L. monocytogenes*, *L. seeligeri*, and *L. ivanovii* was enhanced by the use of HRBC treated with the CE of *R. equi*, *P. fluorescens*, *A. calcoaceticus*, and *S. aureus* (Table 2). The hemolytic activity enhancement was expressed as an increase of CHU or MHU or both. The MHU titer increased even more when the plates were left at room temperature overnight after incubation. Strains classified as *L. innocua*, *L. welshimeri*, *L. grayi*, and *L. murrayi* showed no hemolytic activity on treated HRBC (Table 3).

DISCUSSION

The microplate technique was a simple and reliable method for clearly differentiating between hemolytic and nonhemolytic *Listeria* strains. Therefore, it can be used instead of the CAMP test with *S. aureus* for the identification of the species *L. monocytogenes* and *L. seeligeri*, which can exhibit such weak hemolysis on blood agar plates that the activity could be interpreted as negative. From our point of view, the use of this microhemolysis test for routine typing of *Listeria* strains offers several advantages when compared with the CAMP test with *S. aureus*: it is economical in time, space, and materials because it does not require the growth of bacteria on culture media and strict sterility; because there are no interferences due to the development of bacteria on culture media, a factor that has been proved as an important source of errors (18), the results are more easily standardizable; and it has greater sensitivity than the CAMP test with *S. aureus*, in which false-negative results may be observed, according to our experience (8) and that of other investigators (18).

Furthermore, our results suggest that *L. seeligeri* and *L. ivanovii*, despite having the same sugar acidification patterns, could be differentiated based on quantitation of their hemolytic activity without using the CAMP test with *R. equi*. These species seem to follow a characteristic hemolytic pattern when examined by the microtechnique. *L. ivanovii* displayed intense hemolytic activity, reaching 24 CHU and 384 MHU; on the other hand, *L. seeligeri* strains showed very weak hemolysis (0 CHU and 4.8 MHU), a character which sharply differentiates this species from *L. ivanovii* and *L. monocytogenes* (in questionable cases, we suggest the use of treated HRBC, e.g., with *R. equi* or *P. fluorescens*, to unmistakably show the weak hemolysis by *L. seeligeri*). However, it must be emphasized that a positive CAMP test with *R. equi* is a specific marker which allows the identification of *L. ivanovii* (25) and should be performed in any case of doubt.

With respect to the different hemolytic intensities of *L. monocytogenes* strains, we must point out that this is a well-known phenomenon (30, 31); strains of serovar 3a are recognized as especially hemolytic (14, 27). Two of the *L. monocytogenes* strains which we examined (Se-3a and Se-1/2b) reached hemolytic titers as high as those observed for *L. ivanovii* (24 CHU and 384 and 192 MHU), thus emphasizing the relevance of the sugar acidification tests as phenotypic markers for the differentiation of both species when the microtechnique is used.

On the other hand, our results show that the treatment of HRBC with the CE of *R. equi* produced similar increases in the hemolytic activity of the three *Listeria* species that bear this characteristic. This nonspecific synergistic effect on hemolysis by *Listeria* species was previously demonstrated by other investigators when exosubstances of *R. equi* were added to blood agar medium (30, 31) and has been proposed to differentiate pathogenic from nonpathogenic *Listeria* strains (29). These results seem to be opposed to those obtained when the *R. equi* strain, instead of its exosubstances, was used on blood agar plates; in that case the synergistic effect of *R. equi* appeared to be specific for *L. ivanovii* (13), the hemolysis of which is strongly enhanced and typically shovel shaped (27, 30); this is used as a fundamental criterion for the differentiation of *L. ivanovii* (22). Nevertheless, from our experience and that of other investigators (17, 30), the hemolysis of *L. monocytogenes* is also enhanced when the organism is grown close to *R. equi*, although the effect is smaller and rounded. This indicates that the synergistic hemolytic phenomenon exerted by *R. equi* is not strictly specific for *L. ivanovii* and supports the results obtained when its exosubstances were used.

On the other hand, treatment with the CE of *S. aureus* produced not only an increase in the hemolytic activity of *L. monocytogenes* and *L. seeligeri* but also of *L. ivanovii* strains; these results are not in accordance with those obtained by Skalka et al. when partially purified staphylococcal β-toxin was used (30) or with those reported by other investigators (4, 22, 25) for the CAMP test of *L. ivanovii* with *S. aureus*.

Our results also show that the synergistic effect on hemolysis by *Listeria* species may be observed by using HRBC treated with the CE of the gram-negative bacteria *P. fluorescens* and *A. calcoaceticus*. The fact that these two species produce phospholipase C, as reported for *S. aureus* (16) and *R. equi* (2), seems to suggest that this enzyme sensitizes the membrane of HRBC, making it more vulnerable to *Listeria* hemolysin. Some investigators (30), on the basis that *L. monocytogenes* also produces phospholipase C (14, 15), consider that the synergistic lysis phenomenon may be explained by the cooperative action between two phospholipases, as has been demonstrated between *Corynebac-*

terium ovis and *R. equi* (2). This is also suggested by the fact that the CE of *R. equi*, *P. fluorescens*, and *A. calcoaceticus* produce an increase in the hemolytic activity of the CE of *S. aureus* (Table 1), the β-toxin of which is a sphingomyelinase C. However, because we did not use purified exosubstances, but used culture supernatants in which there may have been several active compounds that could act even nonenzymatically (1), our results are merely orientative. Nevertheless, the aim of the present paper is not to give an explanation of the synergistic hemolytic effect but to propose a reliable method for the analysis and quantitation of hemolysis by *Listeria* strains.

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